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Potentiation of the ADP-induced platelet aggregation by collagen and its inhibition by a tetrahydrothienopyridine derivative (Y-3642)

DURING the experiments on collagen-induced platelet aggregation, it was found that ADP-induced platelet aggregation was potentiated by collagen at a concentration not sufficient to aggregate rabbit platelets.

This experiment was carried out by turbidimetric method[1] using platelet aggregation meter (Evans, EEL 169). All glasswares used were siliconized beforehand. ADP (sodium adenosine diphosphate dissolved in physiological saline. Sigma Chemical Corp.) and collagen (from bovine achilles tendon, Tokyo Kasei Organic Chemicals) were used as aggregating agents. After the addition of 200 mg of collagen to 10 ml of physiological saline, the mixture was homogenized and centrifuged (1000 rev/min, 10 min). The slightly opaque supernatant obtained was used as the aggregating agent (optical density in 660 mμ, OD_{660} = 0·180). Platelet-rich citrated plasma, 0·5–1·0 × 10^8 platelets/ml, was obtained from rabbits by the methods reported in the preceding paper.[2]

To 0·9 ml of platelet-rich citrated plasma, 0·1 ml of various concentrations of collagen was added under stirring (2000 rev/min) with stirrer-rod of the aggregation meter. Turbidity of the mixture was measured at room temperature (about 25°) during 10 min. The platelets did not aggregate with collagen in a concentration of OD_{660} = 0·180 and less.

When 10 μl of 100 μM ADP was added by a microsyringe to 0·9 ml of platelet-rich citrated plasma under stirring, the platelets aggregated. Disaggregation of the platelet aggregates started after about 1·5 min, and was completed after 3 min.

Addition of 0·1 ml of various concentrations of collagen to 0·9 ml of platelet-rich citrated plasma, followed 1 min later with 10 μl of 100 μM ADP to the mixture potentiated the ADP-induced platelet aggregation.

TABLE 1. POTENTIATION OF ADP-INDUCED PLATELET AGGREGATION BY COLLAGEN

Concentration of		Velocity (mm/min) of		Maximal aggregation
Collagen (initial) (OD_{660})	ADP (final) (μM)	Aggregation	Disaggregation	(Change of turbidity) in mm
0·180	0	0	0	0
0·180	1	27·5	0	40·0
0·090	1	27·0	−1·5	30·4
0·045	1	27·0	−3·0	29·0
0·018	1	28·5	−9·3	23·0
0·009	1	29·0	−13·0	21·5
0	1	28·5	−18·0	23·0

Ten μl of 100 μM ADP was added to 0·9 ml of platelet-rich citrated plasma 1 min after the addition of 100 μl of collagen. Results are shown as mean value of duplicated experiments. Each velocity of the aggregation or disaggregation is expressed in mm/min by the method of Baumgartner.[3]

There was no effect of collagen addition on the initial velocity of platelet aggregation, as shown in Table 1, but the degree of maximal fall of turbidity was in proportion to the concentration of collagen added, at a concentration higher than OD_{660} = 0·0180. The velocity of disaggregation was in inverse proportion to the concentration of collagen. Addition of collagen to platelet-rich citrated plasma in a concentration of OD_{660} = 0·180 did not produce disaggregation.

The platelets aggregated slightly by the addition of 10 μl of 25 μM ADP to 0·9 ml of platelet-rich citrated plasma, and then disaggregated soon later. When 0·1 ml of collagen (OD_{660} = 0·090) was added to 0·9 ml of platelet-rich citrated plasma 1 min before the addition of ADP under the same conditions, aggregation of platelets was potentiated by collagen and was irreversible.

The aggregation of platelets used was neither induced by less than 0·1 μM ADP, nor potentiated by the addition of collagen. This potentiation by collagen was observed only when collagen was added to platelet-rich citrated plasma at the same time as or before ADP. This result was reproducible in repeated experiments using different specimen of platelets obtained from other rabbits.

As a similar phenomenon, potentiation of ADP-induced human platelet aggregation by epinephrine was reported by Mills *et al.*[4] As it is necessary for the potentiation by collagen to precede aggregation of the platelets by ADP, a certain concentration of ADP may act as a trigger for the action of collagen. Collagen may accelerate the release of ADP from platelets and itself act as an inhibitor of disaggregation of platelet aggregates *in vitro* or *in vivo*.

2-Amino-3-ethoxycarbonyl-6-benzyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine hydrochloride (Y-3642·HCl) is a new compound having analgesic, antipyretic and anti-inflammatory activities with low toxicity.[5] It inhibited the aggregation of rabbit platelets induced by ADP, thrombin, or collagen and also the release of adenosine nucleotides from rabbit platelets by thrombin.[6]

By a method similar to that described above, 10 μl of Y-3642·HCl (final concentration, 100 μM) or solvent (dimethyl sulfoxide as control) was added to 0·9 ml of platelet-rich citrated plasma and, 1 min later, 10 μl of 50 μM ADP and 0·1 ml of collagen of various concentrations were added to this mixture under stirring.

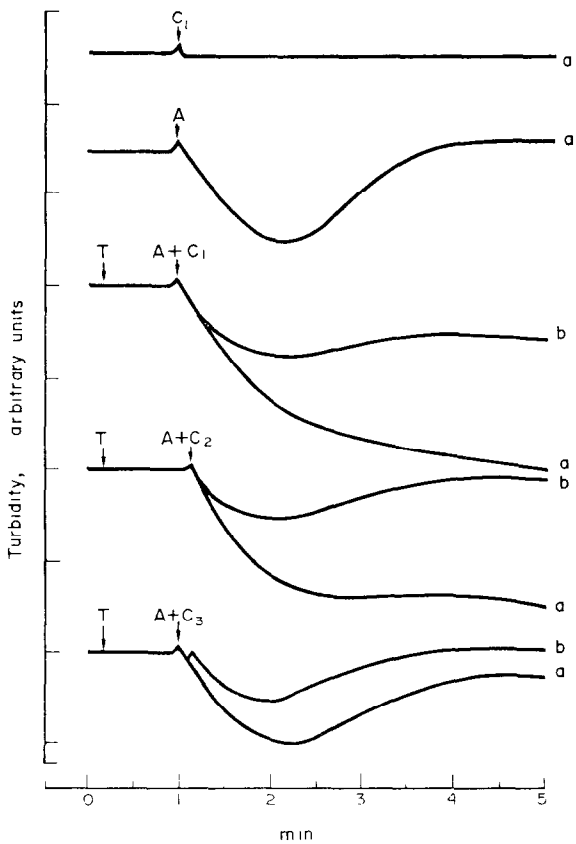

FIG. 1. Effects of Y-3642·HCl on potentiation of ADP-induced platelet aggregation by collagen

C: Collagen, C_1; OD_{660} = 0·090, C_2; OD_{660} = 0·054
C_3; PD_{660} = 0·018
A: ADP (0·5 μM in final concentration)
T: Test solution (b, Y-3642·HCl, 100 μM; a, control).

Y-3642·HCl inhibited the potentiation of ADP-induced platelet aggregation by collagen at this concentration and completely inhibited at a low concentration of collagen (OD_{660} = 0·018), but hardly affected the initial velocity of platelet aggregation, as shown in Fig. 1. The effect of Y-3642. HCl may be due to the suppression of the release of ADP from platelets.

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Inhibition of microsomal mixed-function oxidase activity with nicotinamide*

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UNTIL quite recently, virtually all investigators of hepatic microsomal mixed-function oxidase systems used nicotinamide in their incubation media to inhibit pyridine nucleotidase in an attempt to preserve maximal levels of NADP. This practice was discontinued in most laboratories after Schenkman *et al.*[1] showed that nicotinamide not only failed to enhance, but actually inhibited, the oxidation of certain drugs, probably by competing for the type II binding site of cytochrome P-450. These studies were rigorously controlled with respect to enzyme content, NADPH concentration and incubation time. The authors suggested that the "determination of Michaelis constants (K_m) currently in the literature, for example, for various substrates of the microsomal mixed-function oxidase should be repeated in a more uniform manner, and in the absence of inhibitors like nicotinamide". We were particularly interested in these recommendations because we were responsible for the reporting of a number of these Michaelis constants.[2–5] In those studies we had been mindful of incubation times and microsomal content of the media and were satisfied that reaction rates were proceeding linearly throughout the incubation period. When there was doubt that this was the case, as for example when a relatively high percentage of substrate was oxidized during the period of observation, values were adjusted in the form of an integrated Michaelis constant.[2] Nevertheless, nicotinamide was employed in these studies and, even though the concentration of 4 mM was considerably below the 50 mM concentration used by Schenkman *et al.*, we were concerned as to whether or not nicotinamide might have distorted the kinetic constants we had reported.

The *N*-dealkylation of morphine, ethylmorphine, 3-methyl-4-methylaminoazobenzene (3-MMAB) and 2-diethylaminoethyl-2,2-diphenylvalerate (SKF 525-A) was studied using microsomes (105,000 *g* pellet) prepared as described previously[2] from the livers of male Holtzman strain rats (100–130 g). The incubation medium was the same as that described previously,[2] but it was varied to contain 0, 4 or 50 mM nicotinamide. The incubation mixture contained from 0·6 to 0·8 mg of microsomal protein per ml as determined by the method of Lowry *et al.*,[6] which is equivalent to 50 mg of fresh liver. Uniform reaction rates were observed throughout the various incubation periods, which were 7·5, 10, 15 and 30 min, respectively, when SKF 525-A, 3-MMAB, morphine and ethylmorphine were employed as substrates. Formaldehyde formed from the *N*-demethylation of morphine, ethylmorphine

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